

FIG. 3. Variations in egg number, egg size and total egg volume with changes in altitude and spawning date. Open circle: lowland population, Solid circle: montane population, *: significant at $<.05$, **: significant at $<.01$, a: relationship between egg number and altitude, b: relationship between egg number and date of oviposition, c: relationship between egg size and altitude, d: relationship between egg size and date of oviposition, e: relationship between total egg volume and altitude, f: relationship between total egg volume and date of oviposition.

mean total egg volume varied conspicuously, and a highly negative relationship was observed between the mean total egg volume and the date of oviposition. However, among montane populations, there was a positive correlation between the mean total egg volume and altitude.

The relationship between the mean number of eggs and egg size among populations is shown in Figure 4. Among all the populations, there was no significant correlation between egg number and egg size ($n=32$, $r_s = -0.307$), but, there was a negative correlation between the mean number of eggs and mean egg size among montane popula-

tions ($n=16$, $r_s = -0.738$, $P < .01$). Among the lowland populations, a weakly (not significant) positive correlation ($n=16$, $r_s = 0.413$) was seen between mean egg number and mean egg size.

3. Analysis of variations in the egg number, egg size and total egg volume

As a result of simple correlation, the number of eggs, egg size and total egg volume related in a complex manner with the difference in altitude and the date of oviposition. On the whole, the date of oviposition was significantly correlated with altitude. Among lowland populations, however, the

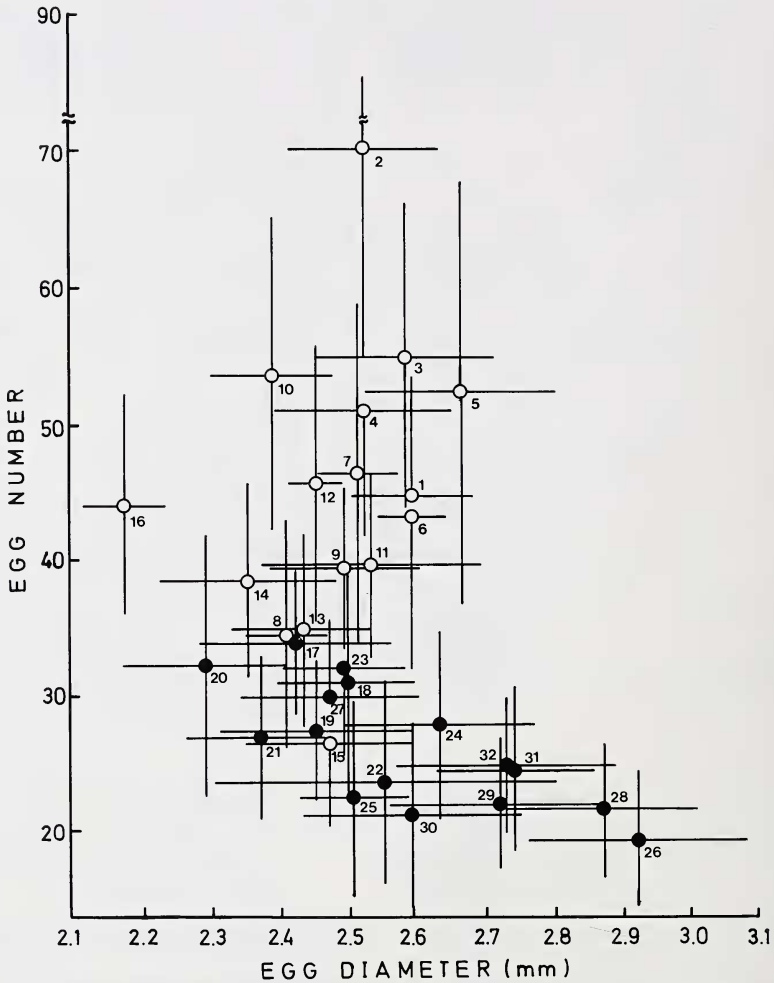


FIG. 4. Relationship between mean egg number and mean egg size. Vertical and horizontal lines: standard deviations, Open circle: lowland population, Solid circle: montane population.

number of eggs, egg size and total egg volume varied independently from altitude. For the above reason we estimated that the altitudinal and breeding-seasonal factors are independent of each other as far as the variation in the number of eggs, egg size and total egg volume are concerned. In order to analyze these variations from the viewpoints of both the effect of altitude and the breeding season, each variation was examined by the multiple regression analysis using altitude and the date of oviposition as predictor variables (Table 2). These regression equations were fairly effective in explaining the variations in the mean number of eggs

and total egg volume. According to the results of the test of significance in the partial regression coefficient in Table 2, both the differences in altitude and the date of oviposition are significant in regard to the variations in the number of eggs and total egg volume among all the populations examined. These regression analyses showed that the mean number of eggs and mean total egg volume increased with altitude, but decreased with the postponement of oviposition. These results also showed that the altitude and the date of oviposition are factors independent of each other.

Variation in egg size cannot be adequately ex-

TABLE 2. Results of multiple regression analyses

Criterion variable	Multiple R	Analysis of variance		Constant ($\times 10^{-2}$)	Predictor variable	Partial regression coefficient	Standard error	Test of significance in coefficient		Standard partial regression coefficient
		F-ratio	P					t-val	P	
a) Analyses to explain each reproductive characteristic by altitude and date of oviposition										
Egg number ($\times 10^2$)	0.857	40.4	<.001	64.7	Altitude	0.86	0.38	2.296	<.05	0.422
					Date of oviposition	-45.70	0.07	-6.476	<.001	-1.190
Egg size ($\times 10^3$)	0.534	5.8	<.01	25.2	Altitude	0.19	0.08	2.404	<.05	0.726
					Date of oviposition	-1.19	1.49	-0.800	>.1	-0.242
Total egg volume	0.810	27.7	<.001	11.6	Altitude	0.30	0.08	3.881	<.001	0.813
					Date of oviposition	-9.56	1.45	-6.616	<.001	-1.386
b) Analysis to explain egg size variation by altitude, latitude and population size										
Egg size ($\times 10^3$)	0.787	15.2	<.001	-69.1	Altitude	0.17	0.04	4.619	<.001	0.657
					Latitude ($\times 10^3$)	0.25	0.07	3.724	<.001	0.503
					Population size	0.11	0.04	2.562	<.05	0.322

plained by the difference in the altitude and the breeding season (Table 2a). According to the results of the test of significance in the partial regression coefficient, altitudinal difference is significant in regard to the variation in egg size among all the populations examined, but the difference in the date of oviposition is not significant. In order to analyze the factor of egg size variation, multiple regression analysis was performed again to use altitude, latitude and population size (number of egg sacs) as predictor variables (Table 2b). This regression was effective in explaining the variation and the test of partial regression coefficient revealed that altitude, latitude and population size are significant factors in egg size variation.

The results of this regression analysis showed that mean egg size increased with altitude, latitude and population size. Similar analyses were performed for the number of eggs and total egg volume. However, latitude and population size were not significant as the variables to explain the variations.

Effectual results which were obtained by linear

regression models on the variations in the egg number, egg size and total egg volume meant that the variations in the egg number, egg size and total egg volume were clinal variations which corresponded with gradual environmental changes.

DISCUSSION

1. Breeding seasonal and altitudinal effects on the number of eggs and total egg volume

We collected many adult specimens of this species in several breeding ponds, and found no evidence of food ingestion at the time. So the active period of this species is estimated to be between the ending of breeding and snowfall in Niigata area. Therefore, delayed breeding shortens the active period of salamanders, and, as a result, a decrease in the total amount of food ingested throughout the year may occur. It is conceivable that total ingestion is one of the factors which restrict total egg volume. Fraser [9] showed experimentally in *Plethodon cinereus* and

Kaplan [10] showed in *Bombina orientalis* that a difference in the nutritional condition of the female affected the process of oocyte growth. Such a phenomenon may be occurring among different populations of *H. nigrescens*. It seems that the decrease in total egg volume is induced by the nutritive deterioration that is brought about by the delay of oviposition, and that the number of eggs decreases to correspond with the decrease in total egg volume.

It has been shown in several species that the altitudinal difference induced variations in life history traits which include variation in the age and size at maturity [6, 11–14]. These variations in life history traits had different patterns according to circumstances. In *Desmognathus ochrophaeus* [11] and *Rana sylvatica* [6], sexual maturation occurred later in montane than in lowland populations, and the body size of females increased with the postponement of sexual maturation. In *Rana pretiosa* [12], sexual maturity was delayed remarkably in a high elevation population compared with a lowland population, but the body size at maturity was similar in both populations. On the other hand, it was estimated that sexual maturity was delayed in high elevation populations, and the body size of females was smaller in high elevation populations than in low elevation ones in *Ambystoma gracile* [13]. These high elevation populations had a 4 months shorter active period than low elevation ones. These reports showed that sexual maturity is reached later in higher altitude populations than in lower altitude ones, and that the growth rate of most females in high altitude populations is reduced by reason of the decrease in the length of the active period in high altitude populations. In northern populations of *Plethodon glutinosus*, growth was greatly retarded owing to a longer hibernating period in comparison with southern populations which did not hibernate [15]. In general, the clutch size and the total egg volume are closely correlated with the body size of females [1, 2, 16]. It is conceivable that the increase in the number of eggs and total egg volume with altitude in the multiple regression analysis is a reflection of the relative increase in female body size resulting from the postponement of sexual maturation. However, if the growth of females is restricted by a

remarkable delay in the breeding season, the body size of adult females is reduced in spite of the delay of sexual maturation. After all, it seems that the relationship between the two variables (the date of oviposition and altitude) in multiple regression equations for the number of eggs and total egg volume means the relationship between the environment effect in growth or vitellogenic process, and the age of maturation of females.

2. Variation in egg size

It has been known that in some amphibian species the egg size is closely related to the altitude [3, 4, 6] or latitude [17, 18]. The present study showed that egg size varied clinally with altitude and latitude. It is thinkable that the egg size variation is related to pond temperature in parallel with altitude and latitude. However, in the breeding ponds examined in the present study, the seasonal variation in water temperature within a pond tended to be greater than geographical variations among ponds (unpublished data). That is, the variation in pond temperature in breeding and hatching is mostly determined by the difference of the date of oviposition among populations. So, we cannot assert that the difference of water temperature based on the altitude and latitude is one of the factors inducing these altitudinal and latitudinal egg size clines.

On the other hand, egg size was significantly related with population size. It seems that the number of egg sacs in one breeding pond is an indicator of the degree of intraspecific competition, of relative food density per larva and of larval density in the breeding ponds. It is conceivable that other pond-specific factors are also involved in egg size determination. However, these factors could not eliminate the altitudinal and latitudinal egg size clines. Populations Nos. 1–5 and 7 were located in the same heap of mountains and at a similar altitude. Furthermore, these populations had a similar breeding period. In spite of the various environmental conditions in their breeding ponds, mean egg size did not vary conspicuously among populations.

3. Relationship among reproductive traits

Berven [6] discussed the relationships among the

number of eggs, egg size, total egg volume, body size and age at first reproduction. He proposed that selection had acted on egg size, and that other reproductive traits had evolved secondarily. Results of the multiple regression analyses in the present study showed that the number of eggs and total egg volume were affected by the date of oviposition. As stated above, it is estimated that the date of oviposition affects the growth of female and the vitellogenic process. Several studies have shown that the differences in growth rate and body size of amphibians were influenced by environment [6, 19, 20]. It is conceivable that the body size of female is one of important factors which determine the number of eggs and total egg volume. Thus, number of eggs and total egg volume in each population appear to be largely environmentally determined. On the other hand, it is conceivable that egg size was not affected by the date of oviposition, but varied in parallel with altitude and latitude. It seems that the egg size varied in adaptation to the altitudinal and latitudinal changes in environment. From the negative correlation between the number of eggs and egg size among montane populations (Fig. 4), it is suggested that high altitudinal populations produce optimal sized eggs at the expense of the number of eggs.

REFERENCES

- Salthe, S. N. (1969) Reproductive modes and the numbers and size of ova in the urodeles. *Am. Midl. Natur.*, **81**: 467-490.
- Salthe, S. N. and Duellman, W. E. (1973) Quantitative constraints associated with reproductive mode in anurans. In "Evolutionary Biology of the Anurans". Ed. by J. L. Vial, Univ. Missouri Press, Columbia, Missouri, pp. 229-249.
- Pettus, D. and Angleton, G. M. (1967) Comparative reproductive biology of montane and piedmont chorus frogs. *Evolution*, **21**: 500-507.
- Kozłowska, M. (1971) Differences in the reproductive biology of mountain and lowland common frogs, *Rana temporaria* L. *Acta. Biol. Crac., Ser. Zool.*, **14**: 17-32.
- Woodward, B. D. (1982) Local intraspecific variation in clutch parameters in the spotted salamander (*Ambystoma maculatum*). *Copeia*, **1982**: 157-160.
- Berven, K. A. (1982) The genetic basis of altitudinal variation in the wood frog *Rana sylvatica*. I. An experimental analysis of life history traits. *Evolution*, **36**: 962-983.
- Kurasawa, J. and Iwasawa, H. (1977) Correlation between the number of eggs deposited in the egg sacs and the altitude of the spawning place in the salamander *Hynobius nigrescens*. *Jpn. J. Herpetol.*, **7**: 27-31. (in Japanese with English summary)
- Takahashi, H. and Iwasawa, H. (1988) Changes in the size of egg-form embryos with the advance of development in the salamander *Hynobius nigrescens*. *Jpn. J. Herpetol.*, **12**: 124-126.
- Fraser, D. F. (1980) On the environmental control of oocyte maturation in a plethodontid salamander. *Oecologia*, **46**: 302-307.
- Kaplan, R. H. (1987) Developmental plasticity and maternal effects of reproductive characteristics in the frog, *Bombina orientalis*. *Oecologia*, **71**: 273-279.
- Tilley, S. G. (1973) Life histories and natural selection in populations of the salamander *Desmognathus ochrophaeus*. *Ecology*, **54**: 3-17.
- Licht, L. E. (1975) Comparative life history features of the western spotted frog, *Rana pretiosa*, from low- and high-elevation populations. *Can. J. Zool.*, **53**: 1254-1257.
- Eagleson, G. W. (1976) A comparison of the life histories and growth patterns of the salamander *Ambystoma gracile* (Baird) from permanent low-altitude and montane lakes. *Can. J. Zool.*, **54**: 2098-2111.
- Tilley, S. G. (1980) Life histories and comparative demography of two salamander populations. *Copeia*, **1980**: 806-821.
- Highton, R. C. (1962) Geographic variation in the life history of the slimy salamander. *Copeia*, **1962**: 597-613.
- Kaplan, R. H. and Salthe, S. N. (1979) The allometry of reproduction: an empirical view in salamanders. *Am. Natur.*, **113**: 671-689.
- Moore, J. (1949) Geographic variation of adaptive characters in *Rana pipiens* Schreber. *Evolution*, **3**: 1-24.
- Matsui, M. (1987) Geographic variations in reproductive traits and the factors of the variations. In "Biology of Toad". Ed. by A. Urano and K. Ishihara, Shokabo, Tokyo, pp. 19-31. (in Japanese)
- Berven, K. A. (1982) The genetic basis of altitudinal variation in the wood frog *Rana sylvatica* II. An experimental analysis of larval development. *Oecologia*, **52**: 360-369.
- Harris, R. N. (1984) Transplant experiments with *Ambystoma* larvae. *Copeia*, **1984**: 161-169.

Thyroid Status and Ambient Temperature as Influences on Weaning in Young Mice

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ABSTRACT—Depressed thyroid status has been long known to delay normal mammalian development of physiological and behavioral processes. Investigations of young rats made hypothyroid by chemical goitrogenesis have demonstrated that such a treatment disrupts normal weaning behavior which can be restored by thyroxine (T_4) injection. The present study was done to determine the influence of chemically-induced hypothyroidism on weaning in the mouse, an experimental rodent closely related to, but somewhat metabolically different from the rat. Hypothyroidism was instituted by incorporation of thiouracil (0.25%) into the maternal diet from the day of mating. Daily from day 6 of life, half the pups in each litter born to euthyroid or hypothyroid dams were injected with a physiological dose (50 ng/g body wt) of T_4 , with the remaining pups receiving vehicle. At 17, 21, or 24 days of age, pups were allowed to indicate a preference between maternal food or non-maternal (chow) food choices at either room (24°C) or incubator (35°C) temperature. After spending the night following preference testing in the home cage, stomach contents of pups were analyzed for proportions of milk and chow. Thyroxine injection of euthyroid pups did not significantly modify body weight, circulating T_4 levels, or behavioral progression to weaning, suggesting the dose to be physiological. Hypothyroidism effectively eliminated weaning behavior within the normal time frame, but the behavior was restored by T_4 injection. Elevation of ambient temperature generally increased pup preference for a non-maternal food source at 21 and 24 days, however the increases were small and did not represent accelerated weaning in euthyroid pups nor normal weaning in hypothyroid mice. In general, the influence of thyroid status on normal weaning in mice appears to be very similar to that reported in rats.

INTRODUCTION

The influence of insufficiency of thyroid hormone on pre- and postnatal developmental processes has been known for some time [1]. However, details regarding the presence or severity of specific developmental deficiencies remain to be investigated in hypothyroid experimental animals. The normal process of weaning from total reliance upon mother's milk has been well established in the rat [2], with the first appearance of solid food in the stomach occurring between days 15-17 [3], and the disappearance of milk between days 24-28 [4]. Recent studies have also found this precisely timed developmental process to be delayed by depressed thyroid status [5, 6] which may result in part from disrupted temperature control mecha-

nisms which occur because of hypothyroidism [5]. To our knowledge, similar studies have not been done in the laboratory mouse, a species in which adult body weight is about 10% of that in the rat [7] and in which metabolic rate is about twice that of the rat [8]. Thus, the present study was carried out to determine whether hypothyroidism delayed weaning in the mouse, and if so, whether replacement of thyroxine (T_4) or elevation of ambient temperature would ameliorate this delay. These determinations were made by conducting dietary preference tests after fasting at 17, 21 or 24 days of age (bracketing the time of normal weaning in rats), and by measuring the proportion of stomach contents comprising milk and solid food the next day, after pups had spent the night with the mother mouse.

MATERIALS AND METHODS

Mice derived from the Swiss-Webster strain were mated, with successful matings being determined by presence of a vaginal plug. On the first day of pregnancy, females were isolated in opaque polystyrene cages under controlled temperature ($22 \pm 10^\circ\text{C}$) and lighting (12L:12D) conditions, and were fed either standard laboratory chow or Lab Blox Mash (Wayne Animal Foods Division, Chicago, Illinois) to which 0.25% by weight 2-thiouracil (Sigma Chemical Company, St. Louis, Missouri) had been added to induce hypothyroidism. The dietary regimen of each pregnant or lactating mouse remained in the same through testing of young around the time of weaning.

On day 5 after birth of each litter, pups in excess of a total litter size of 8 were removed from the nest. The study population consisted of 112 control pups from 18 different litters, and 111 chemically hypothyroid pups from 27 litters. Both male and female animals were used in each experiment. On day 6 of life, and daily thereafter, half the pups in each litter received a physiological dose of thyroxine (T_4 , 50 ng/g body wt) [5] by subcutaneous injection, and the remaining pups were administered an equivalent volume of the hormone injection vehicle (0.3 mM NaOH).

The weaning status of all pups was tested using two measures; a dietary preference test in the absence of the natural mother, and a determination of stomach contents the morning after the dietary preference test, the pups having spent the night in the cage with the natural mother. The dietary preference test essentially followed the regime used by Blake and Henning [5] with young rats. Seventeen, 21, or 24 day old mouse pups were incubated ($35 \pm 2^\circ\text{C}$) and fasted with available drinking water for 10 hr, after which a vehicle-injected and T_4 -injected pair were placed midway between chow mash, chow pellets, and a lactating Nembutal-anesthetized foster dam with nipples exposed at either room (24°C) or incubator (35°C) temperature. Behaviors were catalogued by observation at 10 sec intervals over a total test time of 5 min, with the summed percentage time spent eating mash or pellets used to indicate weaning. After dietary preference testing, pups

were returned to the maternal cage overnight. The next morning (day 18, 22 or 25) pups were decapitated and blood was collected from the severed neck vessels. Serum was extracted by centrifugation and frozen at -20°C for future estimation of T_4 concentrations. After determination of carcass weight to the nearest 0.1 g, stomachs were rapidly excised and iced for 20 min to coagulate milk [5]. Total stomach contents were removed and weighed, then the chow component was weighed alone. The percentage of total stomach contents represented as chow was used as an index of weaning.

Serum T_4 concentrations were determined to measure the effectiveness of thiouracil in depressing thyroid status, and the degree of T_4 replacement affected by injecting the hormone. Serum samples were pooled with regard to maternal diet (with or without thiouracil) and pup injection (vehicle or T_4), but without regard to age, since T_4 levels of normal mice are fairly consistent across this age range [9]. T_4 concentrations were determined using radioimmunoassay kits generously donated by Micromedic Systems, Inc. (Horsham, Pennsylvania). T_4 sensitivity of these kits was 0.3 ng/dl, having a within assay variation of 6.5% and a between assay variation of 7.1%.

Data were statistically analyzed for main effects of thyroid status and ambient temperature upon dietary preference, and of thyroid status on stomach contents and body weight, by analysis of variance, with individual comparisons of means performed using Tukey's test [10]. Differences in serum T_4 concentrations between vehicle- and T_4 -injected pups were evaluated with Student's *t*-test. In all cases significance was ascribed to $P < 0.05$.

RESULTS

The linear growth of euthyroid pups between days 18–25, expressed by the relationship $Y = 2.0X + 4.7$ (slope = 2.0 g/day; $r = 0.87$), was not altered by daily injection of 50 ng T_4 /g body weight, suggesting that this dosage represented a truly physiological level which had largely turned off endogenous thyroid hormone production. This supposition is substantiated by the levels of circu-

lating T₄ which, while slightly elevated in T₄-injected normal animals, was not significantly so (Table 1). Furthermore, administration of this dosage of T₄ increased circulating hormone levels to over 75% of normal in chemically hypothyroid pups, and elevated severely depressed body weights to normal levels.

During the early stages of the weaning process at 17 days of age neither depression of thyroid status with thiouracil nor administration of T₄ by injection to euthyroid pups altered the amount of time spent with the mother or alternate food choices, regardless of the ambient temperature. In all cases, pups spent 2% or less of the test time with food choices alternative to the anesthetized foster dam. However, T₄ administration to thiouracil-fed pups significantly elevated the time spent with non-maternal food options to above 3%, and this influence was augmented to greater than 5% by elevating environmental temperature ($P < 0.05$, Fig. 1). After pups spent the night with the natural mother, analysis of stomach contents revealed that young of euthyroid mice had begun to consume chow at this age, at a percentage of total intake unmodified by T₄ injection (approximately 5% of stomach contents). On the other hand, stomach contents of 18 day old pups from thiouracil-fed dams contained no chow, an observation which was not altered by administration of exogenous T₄ (Fig. 2).

By 21 days of age the depressive influence of thiouracil-induced hypothyroidism on development of weaning behavior had become obvious.

Pups in this group spent less than one-tenth the time with non-maternal food as did normal pups (with or without T₄ injection) or thiouracil-fed animals injected with T₄ (Fig. 1). As at 17 days, elevation of ambient temperature only increased the amount of time spent with non-maternal food by hypothyroid animals injected with T₄. The general trend was for all incubated pups to spend more time away from the foster dam than pups at room temperature, except by normal pups given T₄. On day 22, chow represented over 50% of stomach contents in normal pups, and was not influenced by T₄ injection. While only traces of chow were found in the stomachs of vehicle-injected hypothyroid pups (less than 2% of total contents), T₄ injection resulted in the proportion of chow approximating that seen in normal animals (Fig. 2).

For all intents and purposes, the weaning process was completed by 24 days of age in normal animals, with an average of over 90% of dietary preference test time spent with food sources other than the foster dam. This proportion could be driven to nearly 95% by increasing environmental temperature to 35°C. The completeness of weaning was further substantiated by the presence of 100% chow in the stomachs of these pups on day 25 (Fig. 2). Hypothyroidism continued to delay weaning behavior, with about 5% of the test time being spent with non-maternal food sources, but at this age increased ambient temperature significantly elevated time spent with chow to about twice the average (Fig. 1). Nonetheless, only trace amounts

TABLE 1. Body weights and relative circulating thyroxine (T₄) concentrations of normal and thiouracil-fed mice with or without T₄ injection

Treatment	Injection	Body Weight (g)			T ₄ levels ⁴⁾ (% of control)
		18 days	22 days	25 days	
Lab Chow	Vehicle	6.8±0.1(18) ^{1)a}	8.2±0.1(18) ^d	10.2±0.3(20) ^f	100.0±7.5(12) ^h
	T ₄ ³⁾	6.4±0.1(18) ^a	7.6±0.2(18) ^d	10.5±0.4(20) ^f	117.3±9.9(12) ^h
Lab Chow plus Thiouracil ²⁾	Vehicle	4.8±0.2(18) ^b	6.3±0.2(19) ^e	8.2±0.2(19) ^g	24.0±2.0(12) ⁱ
	T ₄ ³⁾	6.1±0.2(17) ^c	7.8±0.2(19) ^d	10.4±0.2(19) ^f	77.9±4.0(12) ^j

¹⁾ Mean ± SEM(n). Values in the same column with the same superscript are not significantly different ($P > 0.05$).

²⁾ 0.25% of maternal diet by weight from day 1 of pregnancy.

³⁾ 50 ng/g body weight

⁴⁾ 100% T₄ in controls represents a mean concentration of 10 μg/dl.